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Chemotactic and osmotic signals share a cGMP transduction pathway in *Dictyostelium discoideum*

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Abstract In the ameboid eukaryote *Dictyostelium discoideum*, chemotactic stimulation by cAMP induces an increase of intracellular cGMP and subsequently the phosphorylation of myosin heavy chain II. Resistance to high osmotic stress also requires transient increases of intracellular cGMP and phosphorylation of myosin heavy chain II, although the kinetics is much slower than for chemotaxis. To examine if chemotaxis and osmotic stress share common signaling components we systematically analyzed the osmotic cGMP response and survival in chemotactic mutants with altered cGMP signaling. Null mutants with deletions of cell surface cAMP receptors or the associated GTP-binding proteins Gα2 and Gβ show no cAMP-induced cGMP response and chemotaxis; in contrast, osmotic stress induces the normal cGMP accumulation and survival. The same result was obtained with the non-chemotactic mutant KI-10, which lacks the activation of guanylyl cyclase by cAMP. This indicates that these components are required for chemotaxis but not osmotic cGMP signaling and survival. The potential guanylyl cyclase null mutant KI-8 shows no chemotaxis, no osmotic cGMP increase and reduced survival in high osmolarity. Two types of cGMP-binding protein mutants, KI-4 and KI-7, also show reduced tolerance during high osmotic stress. Taken together, these observations clarify that chemotactic and osmotic signals are detected by different mechanisms, but share a cGMP signaling pathway.

Key words: *Dictyostelium*; Osmolarity; Cyclic guanosine 3,5-monophosphate; Chemotaxis; Mutant

1. Introduction

Extracellular folic acid and cAMP attract ameboid *Dictyostelium discoideum* cells. Binding of the chemoattractants to their specific cell surface receptors evokes a chain of intracellular reactions via GTP-binding proteins (G protein), guanylyl cyclase, and cGMP-binding proteins leading to rearrangement of the actomyosin system [1]. The role of intracellular cGMP and its detection by a cGMP-binding protein in the chemotactic signaling was indicated by the observation that nearly all chemotactic mutants are defective in cGMP metabolism or functioning. *Streamer* F mutants show a prolonged chemotactic movement to cAMP, and at the same time exhibit a prolonged cGMP response due to the absence of a cGMP-specific phosphodiesterase [2,3]. Mutant KI-8 lacks nearly all guanylyl cyclase activity and does not respond to any chemoattractant [4]. Guanylyl cyclase in chemotactic mutant KI-10 cannot be activated by cAMP. Finally, mutants KI-4 and KI-7 possess aberrant binding of cGMP to an intracellular cGMP-binding protein (high K_d in KI-4; low K_d in KI-7) [5].

The increase of intracellular cGMP was also characterized as a response to osmotic stimulation [6]. *D. discoideum* wild type cells show a transient accumulation of intracellular cGMP, but mutant KI-8 exhibits no such increase and, at the same time, shows a dramatic reduction of survival in solutions with a high osmolarity [7]. The reduction of survival of KI-8 is restored by addition of a cell permeable analogue, 8Br-cGMP.

Although in both chemotactic and osmotic cGMP signaling the role of cGMP is known to be the trigger for myosin heavy chain II phosphorylation [7,8], which regulates filament self-assembly and thereby controls the interactive forces with actin filaments and cell shape [9], it is not clear yet if the two cGMP signaling pathways share common components. The kinetics of the cGMP response by chemotactic stimulation and by osmotic shock are very different with peaks at 10 s for chemotaxis and at 10 min for osmotic stress [6,7]. In this study we systematically examine the osmotic cGMP response and survival in a large collection of chemotactic mutants, lacking cAMP receptors, its associating transducer proteins Gα2 and Gβ, guanylyl cyclase or a cGMP-binding protein. We demonstrate in this report that chemotactic and osmotic cGMP signal transduction pathways merge into one pathway after the G proteins but before or at guanylyl cyclase.

2. Materials and methods

2.1. Strains and culture conditions

Mutants lacking adenylyl cyclase A (*aca*$^-$) [10], phospholipase C (HD10; *plc*$^-$) [11], cAMP receptors (RI-9; *car*1$^-$/*car*3$^-$) [12], Gα2 (MP-2; *g*α2$^-$) [13] and Gβ (LW-6; β$^-$) [14], KI mutants [4], and the parental strain XP55 were grown on 1/3 SM plate (0.3% glucose, 0.3% bactopeptone, 1.5% agar and 40 mM KH_2PO_4/Na_2HPO_4, pH 6.0) with *Escherichia coli* B/r. Cells were harvested in the late logarithmic phase with 10 mM KH_2PO_4/Na_2HPO_4, pH 6.5 (PB). Bacteria were removed by repeated centrifugation at 300×*g* for 3 min. Cells were starved for 5 h by shaking in PB at a density of 10^7 cells/ml at 21°C.

2.2. Determination of cGMP

To measure cGMP responses to the chemoattractant cAMP and to osmotic stress, cells were stimulated with 10 μM cAMP or 300 mM glucose as described before [7]. Determination of the cGMP concentration was performed by radioimmunoassay as described [15].

2.3. Survival of XP55 and mutants

Measurement of survival was performed with the same protocol as in [7]. Briefly, cells were resuspended in PB at a density of 10^7 cells/ml. Then cells were stimulated with 300 mM glucose and the treatment was terminated by 1000-fold dilution of cells. Survival was measured

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Abbreviations: cAMP, cyclic adenosine 3,5-monophosphate; cGMP, cyclic guanosine 3,5-monophosphate; IP_3, D-myo inositol 1,4,5-trisphosphate

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by plating 200 cells on 1/3 SM plate with *E. coli* B/r and the number of emerging colonies was determined several days later. The survival after osmotic stress is presented as percent of observed colonies relative to untreated cells. The osmotic cell viability was identical when cells were diluted 1000-fold at once or slowly in three 10-fold dilution steps, suggesting that the cells die during osmotic shock, not in the following dilution (data not shown). The survival of untreated cells is not significantly different between the wild type and the mutants used in this study.

3. Results and discussion

3.1. The second messengers cAMP, IP_3 and cGMP

As previously shown, the intracellular cGMP concentration increases in chemotactically stimulated wild type cells with a maximum after 10 s (Fig. 1A). The drastic increase of the osmotic potential to 300 mM glucose has no effect on cGMP levels for at least 2 min, but then results in a large increase of the intracellular cGMP concentration with a maximum at 10 min (Fig. 1B). Wild type cells resist high osmotic stress for about 30 min, followed by a slow decline of survival upon longer exposure to high osmotic stress (Fig. 1C).

In *D. discoideum*, binding of the chemoattractant cAMP to its cell surface receptor results in transient increases of at least two other intracellular second messengers, cAMP [16] and IP_3 [17]. To examine the role of these second messengers in the osmotic regulation, survival and cGMP responses were measured in mutants *aca*$^-$ and *plc*$^-$ with disruptions of the adenylyl cyclase A [10] and phospholipase C genes [11], respectively, and in mutant KI-8 lacking nearly all guanylyl cyclase activity [4]. Whereas mutants *aca*$^-$ and *plc*$^-$ show the normal cGMP response and survival in high osmotic stress (Fig. 2, detailed data not shown), no cGMP response can be observed in mutant KI-8 and these cells rapidly die at high osmotic stress (Fig. 1). The absence of an essential role of adenylyl cyclase and phospholipase C in osmo-sensory transduction is also suggested by the observation that high osmotic stress has no effect on the levels of intracellular cAMP [6] and IP_3 (unpublished data by H.K. and P.J.M.v.H.).

These results suggest that guanylyl cyclase but not adenylyl cyclase or phospholipase C plays a crucial role in the resistance to high osmolarities and is a component shared between chemotactic and osmotic signaling pathways.

In chemotactic cGMP signaling, cell surface receptor and its transducer G proteins participate in the activation of guanylyl cyclase [18].

3.2. Surface cAMP receptors and G proteins

Cell surface cAMP receptors are expressed as four subtypes during the course of development of this microorganism. cAMP receptor 1 (cAR1) is expressed in the first 10 h of development, cAR3 appears between 5 and 15 h, and cAR2 and cAR4 are expressed after 10 h of development [19]. In our assay condition with 5 h starved cells, a double knock-out mutant (*car*1$^-$/*car*3$^-$) ensures that no cAMP receptors are present on the cell surface.

It was shown previously that *car*1$^-$/*car*3$^-$ cells show no increase of cGMP levels upon cAMP stimulation (Fig. 2). On the other hand, the rapid increase of extracellular osmotic

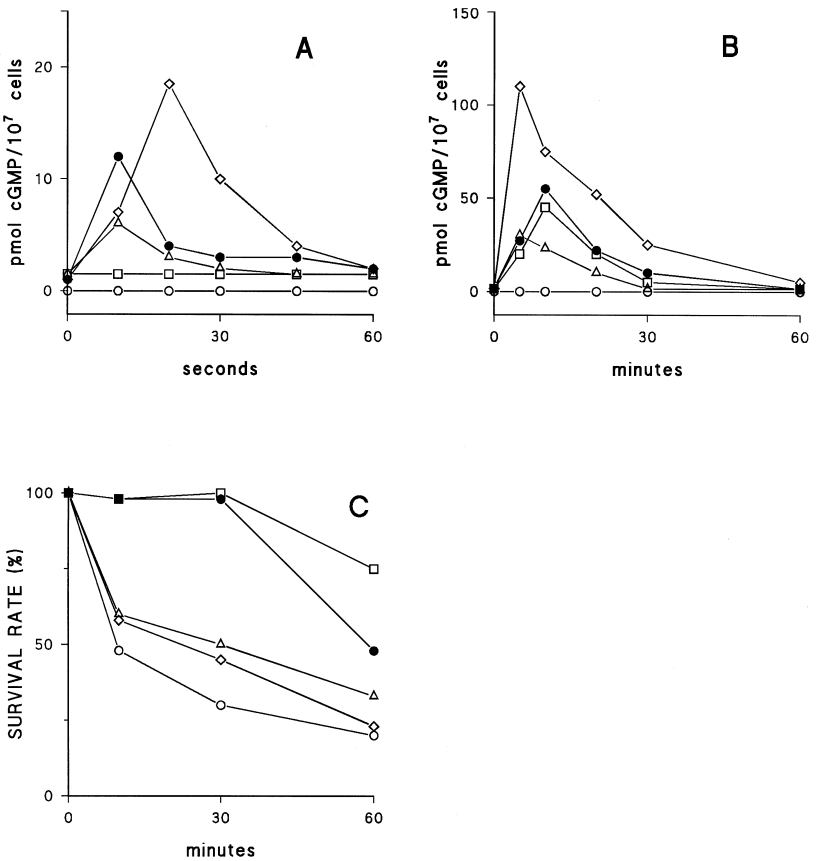

Fig. 1. Time course of cGMP accumulation by cAMP stimulation (A), by osmotic stimulation (B) and viability in high osmolarity (C) of wild type XP55 (solid circle), KI-8 (open circle), KI-4 (open triangle) and KI-7 (open square). cAMP or glucose was added to cells at time 0 to give a final concentration of 10 μM and 300 mM, respectively. All the data are shown as the means of three independent experiments.

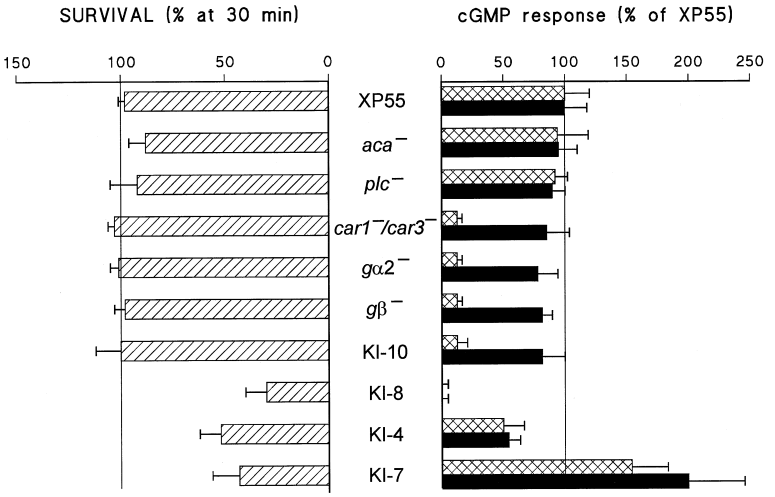

Fig. 2. Relative cGMP accumulation by cAMP stimulation (right; cross-hatched bar) and osmotic stimulation (right; filled bar), and survival (left; hatched bar) of wild type XP55 and the mutants, adenylyl cyclase A null (*aca*$^-$), phospholipase C null (*plc*$^-$), cAMP receptor null (*car*1$^-$/*car*3$^-$), Gα2 null (*g*α2$^-$), Gβ null (*g*β$^-$), KI-10, KI-8, KI-4 and KI-7. The data on the right are presented as the maximum cGMP amount at peak level relative to the amount in XP55 by 10 μM cAMP or by 300 mM glucose stimulation, respectively. The data on the left are presented as % survival at 30 min after adding 300 mM glucose as the final concentration. All the values are the means ± SD of three independent experiments with triplicate determinations.

potential to 300 mM glucose leads to a strong increase of the intracellular cGMP concentration up to the same level and with the same time course compared to wild type cells (Fig. 2). In addition, this mutant resists the high osmolarity to the same extent as wild type cells.

GTP-binding proteins transmit the signal from chemotactic receptors to guanylyl cyclase [18]. The single Gβ subunit is essential for all chemotactic signals [14], whereas Gα2 and Gα4 specifically transduce signals from the cAMP and folic acid receptors, respectively [19]. Knock-out mutants of Gα2 and Gβ were examined for their responses to osmotic stress. The results show that these mutants exhibit the same characteristics as wild type cells (Fig. 2). Taken together, these observations reveal that the cAMP receptors Gα2 and Gβ are not involved in the osmotic cGMP signaling. It is generally thought that Gα subunits cannot function in the absence of a Gβ subunit, suggesting that osmo-sensory transduction to cGMP responses and survival are G protein-independent.

3.3. Osmotic responses in the non-chemotactic mutant KI-10

KI-10 was characterized as a mutant with a deficiency in the coupling of receptor/G protein to guanylyl cyclase [4]. This mutant contains functional receptors and G proteins, since receptor-mediated activation of adenylyl cyclase is essentially normal. Fig. 2 demonstrates that osmotic stress induces the activation of guanylyl cyclase in mutant KI-10 to comparable levels as in wild type cells. KI-10 cells are also resistant to osmotic stress for survival. These results indicate that KI-10 is specifically defective in the activation of guanylyl cyclase via chemoattractant receptors.

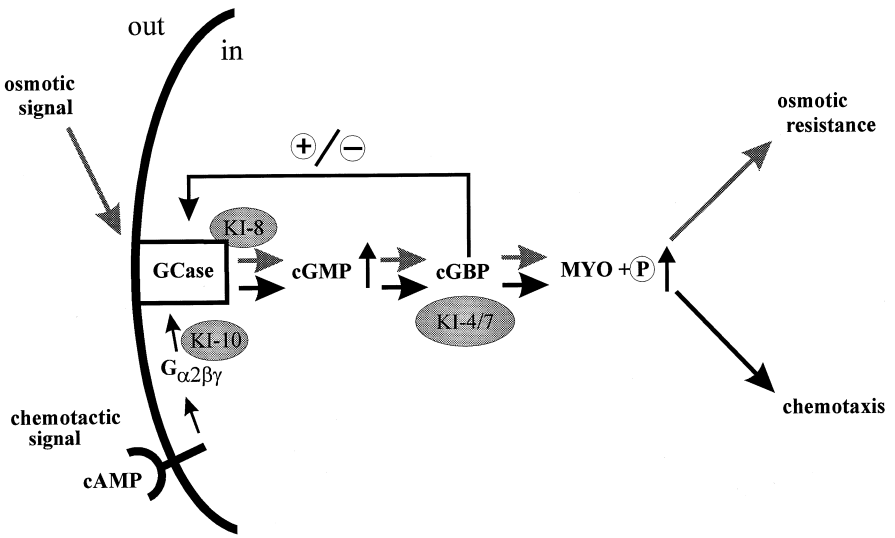

Fig. 3. Model of chemotactic and osmotic signal transduction pathways. cAMP binds to cell surface receptor that interacts with Gα2βγ complex. This signal and the osmotic signal merge into one pathway before or at guanylyl cyclase (GCase). The produced cGMP binds to a cGMP-binding protein (cGBP). This binding protein indirectly induces the phosphorylation of myosin heavy chain II tail region (MYO-P); it also regulates guanylyl cyclase in positive and negative feed-back loop shown in the figure.

3.4. Osmotic responses in non-chemotactic mutants, KI-4 and KI-7

Previously we have demonstrated that the cGMP-binding activity, which shows aberrant properties in KI-4 and KI-7, plays a crucial role in chemotaxis [4]. Furthermore, the cGMP-binding protein functions as a positive and negative regulator of guanylyl cyclase [5]. Accordingly, KI-4 possesses a high affinity cGMP-binding protein, lacks the potentiation of receptor-stimulated guanylyl cyclase, and shows a small cGMP response (Fig. 1A). The cGMP-binding protein of KI-7, in contrast to KI-4, has a low affinity, lacks the inactivation of receptor-activated guanylyl cyclase, and the mutant shows a large cGMP response to cAMP (Fig. 1A).

Osmotic stress activates guanylyl cyclase in mutants KI-4 and KI-7 (Fig. 1B). The relative magnitudes of the responses to osmotic stress and chemoattractant cAMP are similar: large responses in KI-7 and diminished responses in KI-4. In contrast to the significant cGMP responses to osmotic stress, the survival rates of both mutants are strongly reduced (Fig. 1C). These results show that the cGMP-binding protein is an essential component of the osmotic signal transduction pathway.

4. General conclusion

The results are summarized in a model for the chemotactic and osmotic cGMP signaling pathway (Fig. 3). Chemoattractant receptors and G protein mediate guanylyl cyclase activation for chemotaxis but are not essential for the activation of guanylyl cyclase by osmotic stress. This conclusion is consistent with the previous observation that in high osmotic condition wild type cells still respond to chemotactic stimulation to give a further increase of intracellular cGMP levels [6]. This indicates that chemoattractant receptors and G proteins are neither detectors nor transducers in osmotic signal transduction. Recently a MAP kinase kinase (DdMEK) was suggested to be essential for cAMP-mediated activation of guanylyl cyclase [20]. Osmotic activation of guanylyl cyclase is normal in the null mutant, suggesting that DdMEK is not a common component of osmotic and chemotactic signal transduction. In contrast, guanylyl cyclase and the cGMP-binding protein are essential for both chemotaxis and osmotic signal transduction. The cGMP-binding protein is likely to be a protein kinase that phosphorylates and activates a myosin kinase leading to the phosphorylation of myosin heavy chain II [5]. Previously we have demonstrated that this myosin phosphorylation is essential for survival of *Dictyostelium* cells in high osmotic stress.

The measurable Mg^{2+}-dependent guanylyl cyclase activity in vitro exists in the membrane fraction in *Dictyostelium* [21]. Therefore, a physical change of the plasma membrane after osmotic shock may cause a structural change and activation of guanylyl cyclase. These changes should be transient, because at 60 min after osmotic shock cGMP levels have declined to basal levels; at this moment chemoattractants are still able to activate guanylyl cyclase [6]. Alternatively to the activation of guanylyl cyclase by physical modification of the plasma membrane, osmotic stress may be detected by a sensor protein that activates guanylyl cyclase. In bacteria and yeast, a two-component system containing a histidine kinase cascade is indispensable for sensing the osmotic signal [22]. Recently, a member of the histidine kinase family (DokA) was isolated in *Dictyostelium* and its function was analyzed in a null mutant [23]. Even though the null mutant shows a drastic decline in viability upon exposure to high osmotic solutions, the cGMP responses in chemotaxis and osmotic resistance are regulated as in wild type, indicating that this histidine kinase pathway runs either downstream of cGMP production or in a parallel pathway.

In the pathway discussed here, myosin heavy chain II plays a central role. Recently, a double *Dictyostelium* mutant which lacks two F-actin crosslinking proteins, α-actinin and gelation factor, was reported to exhibit an increased sensitivity to osmotic stress [24]. This suggests that actin filaments participate in osmotic resistance, in accordance with the translocation of actin to the cell cortex region during osmotic stress [7]. Furthermore, it was reported that the formation and translocation of small osmolytes may also provide osmotic resistance [25]. These observations indicate that osmotic response is rather complex and may include pathways other than cGMP-myosin phosphorylation pathway.

The primary structure of *Dictyostelium* guanylyl cyclase is not known yet. The characterization of the guanylyl cyclase gene is expected to provide important information on the mechanism how the enzyme is activated by receptor/G proteins and by osmotic stress.

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